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Recipient Cell Nuclear Factors are Required for Reprogramming by Nuclear Transfer

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Summary

Nuclear transfer allows the reprogramming of somatic cells to totipotency. The cell cycle state of the donor and recipient cells, as well as their extent of differentiation, have each been cited as important determinants of reprogramming success. Here we have used donor and recipient cells at various cell cycle and developmental stages to investigate the importance of these parameters. We found that many stages of the cell cycle were compatible with reprogramming as long as a sufficient supply of essential nuclear factors, such as Brg1, were retained in the recipient cell following enucleation. Consistent with this conclusion, the increased efficiency of reprogramming when using donor nuclei from embryonic cells could be explained, at least in part, by reintroduction of embryonic nuclear factors along with the donor nucleus. In contrast, cell cycle synchrony between the donor nucleus and the recipient cell was not required at the time of transfer, as long as synchrony was reached by the first mitosis. Our findings demonstrate the remarkable flexibility of the reprogramming process and support the importance of nuclear transcriptional regulators as mediators of reprogramming.

Introduction

The generation of cloned animals by nuclear transfer has demonstrated that the differentiated state of a cell can be reprogrammed to a more embryonic character and that developmental potential can be fully restored (Gurdon, 1962; Wilmut et al., 1997). This reprogramming process has been reported to depend on the cell cycle stage of the recipient cell into which the nucleus is injected. Reprogramming succeeds when a variety of terminally differentiated nuclei are injected into an unfertilized, metaphase-oocyte (Eggan et al., 2004; Hochedlinger and Jaenisch, 2002). In stark contrast, when a fertilized zygote in interphase is used as a recipient cell, only a zygotic nucleus from another embryo, or the nucleus from a 2-cell stage embryo can support development (McGrath and Solter, 1984). When more differentiated nuclei are introduced into the zygote, development fails (Gao et al., 2002; McGrath and Solter, 1984; Robl et al., 1987; Tsunoda et al., 1987). Although this difference in reprogramming capacity between the

oocyte and the zygote is well documented, there remains little mechanistic clarity as to why recipient oocytes in M-phase can support developmental reprogramming while zygotes in interphase cannot.

A variety of explanations for the cell-cycle dependence of successful reprogramming have been proposed. Developmental failure after nuclear transfer into non-metaphase recipient cells has been attributed to an unidentified reprogramming activity that exists only in the metaphase cytoplasm of unfertilized oocytes and that is degraded following fertilization (Eckardt et al., 2005; Tani et al., 2003). Conversely, it could be that following fertilization an inhibitor of reprogramming becomes expressed. These models are attractive as they could explain why zygotes function as recipients for totipotent nuclei, but do not support development when more differentiated nuclei are used.

It has also been considered that the developmental failure of embryos generated by somatic cell nuclear transfer into zygotes could be due to the amount of time available for reprogramming. In the mouse, zygotic genome activation (ZGA) occurs at the two-cell stage. Following zygotic nuclear transfer there might be insufficient time to prepare a differentiated nucleus for the genome-wide activation of embryonic gene expression (Boiani et al., 2002; Campbell et al., 1996b; Kikyo and Wolffe, 2000; Solter, 2000). Similar to the remodeling of the sperm after fertilization, the somatic cell nucleus would need to be biochemically remodeled in the unfertilized oocyte (Rideout et al., 2001).

Additionally, it has been suggested that developmental failures after zygote nuclear transfer could result from incompatibilities between the recipient and donor cell-cycles that in turn lead to chromosome damage (Wakayama et al., 2000). In this model, cellular processes that are ongoing in the interphase zygote, such as DNA synthesis, might preclude the successful incorporation of incoming donor chromosomes. If this were the case, the arrested nature of the meiotic oocyte might allow for synchronization of the embryonic and somatic cell cycles, allowing successful incorporation of the differentiated donor cell chromosomes.

Interestingly, we have recently demonstrated that the interphase zygote regains its capacity to reprogram a differentiated somatic nucleus when it enters into the first embryonic mitosis (Egli et al., 2007), and regains it again when it enters the mitosis

between the two and 4-cell stage (Egli et al., 2009). These experiments have lead us to propose a new model in which reprogramming factors are not destroyed following fertilization, but are instead sequestered in the nucleus during interphase and widely distributed in the cytoplasm during mitosis (Egli et al., 2008). This model predicts that enucleation of a recipient zygote in interphase would deplete factors required for reprogramming (Egli et al., 2008; Egli et al., 2007) and that depletion of these factors, rather than other difficulties, might be the cause of developmental arrest.

To test our model, and to investigate other proposed mechanisms for failures in reprogramming, we have performed nuclear transfer using donor and recipient cells of various cell cycle states and developmental identities. From these studies we conclude that the retention of critical embryonic nuclear factors within the reconstructed embryo is indeed one of the most significant determinant of whether reprogramming succeeds. Beyond this, we find there is remarkable flexibility in the parameters for successful nuclear transfer.

Materials and Methods

Mice and cell lines

BDF1 mice used as zygote donors were obtained from Charles River laboratories. Transgenic ES expressed the H2B-cherry fusion gene under the control of the combined CMV-chicken beta actin promoter (pCAGGS-H2B-cherry). Tail tip fibroblasts used for nuclear transfer were obtained from adult B6jcBA-Tg (Pou5f1-EGFP)2Mnn/J mice. H2B-GFP transgenic mice were made by integration of pCAGGS-H2B-GFP puro^r plasmid into V6.5 mouse ES cells and injection into blastocysts followed by embryo transfer into pseudopregnant recipient females. ES cells were synchronized in mitosis using 0.1µg/ml nocodazole.

Manipulations

Chromosome transfer into mouse zygotes in mitosis

Manipulations were performed as described previously (Egli et al., 2007), with some modifications. To minimize the amount of spindle material removed, mitotic zygotes were released from the nocodazole block (0.1µg/ml), for 1-3 minutes at 37deg. to induce the formation of a small prometaphase spindle. Zygotes were then manipulated in drops containing 0.02 -0.03µg/ml nocodazole, either at room temperature or at 37deg. to remove the zygotic genome. We observed that low concentrations of nocodazole slow down mitotic progression, but do not depolymerize the spindle, and reduce the amount of spindle material removed from the zygote. If placed directly into low nocodazole concentrations at interphase, zygotes arrested in early prometaphase as seen by the disorganized configuration of chromosomes and a weak signal of microtubule birefringence (see Fig. S4 in the Supplementary Material). Such arrested zygotes have a spindle visible by light microscopy. In the presence of MG-132 as previously used (Egli et al., 2007), zygotes proceed from prometaphase to metaphase within approx. 30-60 minutes and assemble a very robust spindle that becomes increasingly more difficult to remove. Unlike MG-132 that has a toxic effect upon prolonged exposure, nocodazole is not toxic and embryos can be incubated in nocodazole after transfer for recovery for an additional 30 min. This method allows working with a single nocodazole concentration to both arrest and manipulate zygotes throughout the experiment. The use of nocodazole alone instead of in combination with Mg-132 resulted in cleavage with less fragmentation.

Nuclear transfer into zygotes in interphase

Nuclear transfer was carried out 24-28h post hCG. Zygotes were incubated in 0.5µg/ml nocodazole and 5-10 µg/ml cytochalasinB for complete and specific enucleation. Specific enucleation was done using a conical needle with an opening of approx. 2µm, according to (Greda et al., 2006). ES cell genomes were directly injected, while blastomere nuclei were fused by electrical pulse 1.6kV/cm in fusion medium using the LF101 device.

To transfer mitotic zygotic genomes into zygotes enucleated in interphase, mitotic zygotes were selected within a cohort of zygotes and their genome was transferred into a zygote enucleated in interphase from the same cohort (28-33h post hCG).

To break the nuclear envelope of one of the two interphase nuclei of the zygote, the nucleus was removed, broken with a brief piezo-electric pulse and then reinjected into the same zygote.

For isolation of mitotic genomes from 8-cell stage embryos, they were incubated in nocodazole for mitotic arrest at approx. 60h post hCG for 5 hours. Within 20-30min after the release from the nocodazole arrest, mitotic spindles formed that could be aspirated into a needle with a 10µm diameter and injected into an interphase recipient zygote.

Immunohistochemistry

Zygotes and preimplantation stage embryos were fixed in 4% paraformaldehyde over night at 4°C, permeabilized in PBS with 0.5% Triton-X100 (PBS/T) for 20min., blocked in blocking solution consisting of 0.1% PBS/T with 10% FBS over night at 4°C, incubated in primary antibody at 4°C in blocking solution, then washed for 1h at room temperature in 0.1% PBS/T, incubated with secondary conjugated antibody in 0.1% PBS/T at room temperature for 1h, washed for 1h, stained with Hoechst 33342 or Draq5 for 5-10 minutes and used for confocal imaging. Oct4 antibody (Santa Cruz, sc5279) and Cdx-2 antibody (Biogenex) was used at a concentration of 1:200, and BRG1 antibody (Santa Cruz sc-10768) was used at a concentration of 1:50. Conditions were maintained between different samples. Relative quantification of BRG1 staining was done by integrating pixel density in Adobe Photoshop. For BrdU incorporation, embryos were incubated in BrdU (Amersham Cell Proliferation Kit) at 32h post hCG, after the completion of zygotic mitosis. Embryos were fixed with 4% PFA at the 2-cell stage, 45h post hCG, incubated in BrdU primary antibody and DNase according to the manufacturer's instructions.

Microarray analysis

Groups of 20-30 embryos were used for RNA isolation. Total RNA was isolated using PicoPure RNA isolation kit (Molecular Devices), and RNA was amplified by two rounds of T7 transcription using the Illumina TotalPrep RNA Amplification Kit. Amplified RNA was hybridized to the Illumina® Sentrix Mouse Expression BeadChip® RefSeq 8 v2.0

or v1.1 and read by the Illumina Bead Array Reader. Analysis was done using the Illumina Genome Studio Program. Genome-less embryos were generated by removal of the zygotic genome in mitosis followed by mitotic exit stimulated by purvalanolA and 6-DMAP. Primary data will be available at <http://www.ncbi.nlm.nih.gov/geo/>.

Results

Interphase zygotes are suitable as recipient cells

Our observation that nuclear transfer into mitotic oocytes and mitotic zygotes succeeds but that nuclear transfer into interphase zygotes fails, has re-raised the question of why interphase recipient cells are not suitable for reprogramming (Egli et al., 2007). We initially considered the hypothesis that ongoing processes within the interphase recipient cell damage the incoming donor chromosomes, resulting in compromised development (Wakayama 1999).

If it were correct that ongoing processes in the cytoplasm of the interphase zygote invariably damaged incoming donor chromosomes, then it would stand to reason, that this damage to the donor chromosomes would occur even if the recipient cell nucleus were not removed. To test whether an intact interphase zygote could accept incoming donor chromosomes, we injected mitotic ES cell chromosomes and observed whether the resulting embryos could develop to the blastocyst stage.

We opted to use ES donor cells as they could be efficiently micro-manipulated and were reliably arrested in mitosis using nocodazole. By synchronizing the donor cell population in one phase of the cell cycle, we could more easily isolate and study the importance of the recipient's cell cycle status. In these experiments, we tracked the recipient and donor cell chromosomes by using recipient zygotes that expressed the histone H2B-green fluorescent fusion protein (H2B-GFP) and donor cells that expressed the H2B-cherry red fluorescent protein (Fig. 1A,B). Thus recipient cell chromosomes were fluorescent green and incoming donor chromosomes fluorescent red.

Following injection of mitotic donor chromosomes into interphase zygotes, we observed that the incoming chromosomes were not organized into a new nucleus and instead were maintained as condensed chromatin separate from the two parental

pronuclei (see Fig. S1 in the supplementary material). Despite this asynchronous configuration of the chromosomes, the zygotic cell-cycle proceeded normally. These tetraploid embryos entered the first mitosis and underwent the first cleavage division with the same timing as un-manipulated control embryos. At 31h post hCG, 16 of 28 nuclear transfer zygotes (57%) and 23 of 44 (52%) control zygotes had entered mitosis and undergone cell division (Fig. 1 D-G). Following nuclear transfer, interphase zygotes cleaved and developed to the blastocyst stage very efficiently (Fig. 1 H,I). As these tetraploid embryos contained both the donor and recipient chromosomes, they expressed both H2B-GFP and H2B-cherry (Fig.1 G-I). These results demonstrate that contrary to previous suggestion (Wakayama et al., 1999), the interphase zygote can successfully incorporate donor cell chromosomes following nuclear transfer.

Our observations demonstrate that the interphase recipient zygote does not always destroy the donor chromosomes, as was previously observed with somatic interphase donor nuclei (Wakayama et al., 2000). In those cases, it is possible that chromosomal damage to the interphase-donor genomes might have been due to a failure to complete DNA replication prior to entry into mitosis. Consistent with these results, we found that interphase ES cells injected into interphase zygotes most commonly lead to cell cycle delay and developmental arrest (see Table S1 in the supplementary material). In contrast, when we used mitotic donor chromatin, DNA replication was already complete at the time of transfer. This may have allowed the asynchronous cell cycles of the donor and recipient cells to become synchronized at the first mitosis, enabling normal chromosome segregation to proceed.

Despite the successful incorporation of mitotic donor chromosomes into an intact interphase zygote, when mitotic ES cell chromosomes were injected into enucleated zygotes, development still failed (Fig. 1E, supplemental Table 1). These results suggest that something about the interphase enucleation process renders the interphase zygote incapable of supporting reprogramming.

Interphase pro-nuclei contain reprogramming activities

Our observation that interphase zygotes can successfully incorporate incoming donor chromosomes suggested that the difficulties in using these recipient cells might be related to the means by which, or time at which, they were enucleated. To determine whether this was the case, we transferred mitotic donor chromosomes into the interphase zygote and then removed the recipient chromosomes at various times to note the effect. We primarily studied two time points for extraction of the mitotic chromosomes. Following mitotic chromosome transfer, we either enucleated the recipient zygote before the end of the first interphase, or we waited 1 to 5 hours longer and removed the condensed recipient cell chromosomes after entry into the first mitosis.

When mitotic chromosomes were injected into the interphase zygote and recipient cell enucleation was subsequently performed in interphase, development failed at the 2-cell stage (Fig. 1E) (see Table S1 in the Supplementary Material).

To confirm that recipient cell enucleation in interphase was indeed the cause of developmental arrest, we again performed mitotic chromosome injection during interphase but then waited for the transferred embryos to enter the first mitosis, and only then removed the recipient cell chromosomes. So that we could be certain we had distinguished the donor and recipient chromosomes from one another in these experiments, we prevented congregation of the chromosomes onto the first metaphase spindle by incubating nuclear transfer embryos in the presence of nocodazole and used the fluorescently tagged histones described above to allow for visualization of donor and recipient chromosomes (Fig. 1D).

Interestingly, we observed that in 20/20 nuclear transfer zygotes, tagged histones of both colors began to exchange between the donor and recipient cell chromosomes. This suggested that rapid exchange of core histones was taking place in a reciprocal manner. This exchange was reminiscent of the dynamic localization of histone H2B in mouse ES cells described previously (Meshorer et al., 2006). It could be that this incorporation of zygotic histones into donor-cell chromatin contributes to the reprogramming process.

Despite the considerable histone exchange we observed, it was always possible (20/20 zygotes) to unambiguously distinguish between the recipient and donor chromosomes based on the relative intensity of their fluorescence (Fig. 2D). The donor

genome always showed higher relative red fluorescence, while the recipient genome always had higher relative green fluorescence (20/20 zygotes). This difference in fluorescence intensity allowed for the specific and reliable removal of only the zygotic recipient cell chromosomes, while leaving behind the donor chromosomes (Fig. 1J,K). Following extraction of the recipient cell chromosomes in mitosis, nocodazole was removed from the culture medium to allow a spindle to assemble around the donor chromosomes. Following spindle assembly, cleavage proceeded normally. In the newly formed interphase nuclei of the resulting 2-cell embryos, a strong H2B-GFP signal was detected (Fig. 1L) suggesting that histones present in the cytoplasm of the zygote continued to be incorporated into the newly introduced donor cell chromatin.

We found that preimplantation development of embryos created in this way, by interphase chromosome transfer and subsequent mitotic extraction of the recipient chromosomes, was very efficient (Fig. 1L-O) and allowed the derivation of ES cell lines from the resulting blastocysts (Supplementary Fig. 2A-C)). During the development of these nuclear transfer embryos, H2B-GFP green fluorescence was gradually lost from the embryo, while H2B-cherry red fluorescence began to increase after the 4-cell stage. This loss of green fluorescence and increase in red fluorescence indicated that the zygotic chromosomes carrying the H2B-GFP transgene had indeed been successfully removed and that only the donor cell chromosomes remained (Fig. 1M,N).

These experiments confirm that chromosome transfer into an interphase recipient cell is indeed acceptable and that incoming chromosomes are not damaged, even after removal of the recipient cell chromosomes. However, if reprogramming and development are to succeed, the recipient cell chromosomes must either not be removed at all or removed only in mitosis.

Normal chromosome segregation and DNA replication after interphase enucleation

We next sought to determine why nuclear transfer embryos arrest following interphase enucleation but not following mitotic enucleation. One possible explanation for these failures is that when zygotes are enucleated in interphase, factors required for either development or reprogramming are removed. If this were the case, then it must be that these factors are not removed or depleted when zygotes are enucleated in mitosis. This is

a reasonable proposal as many protein factors within the cell localize specifically to the nucleus in interphase but are more widely distributed in the cytoplasm during mitosis (Egli et al., 2008). These factors could include but are not necessarily limited to transcriptional regulators, components of the DNA replication machinery and factors involved in chromosome segregation or cytokinesis. Depletion of any one of these important cellular components might be sufficient to lead to the developmental arrest that we, and others, have observed.

We first considered the possibility that enucleation during interphase could be preventing further development by depleting limiting components of the spindle apparatus or cell division machinery. Undergoing cell division in the presence of insufficient quantities of these components could lead to improper chromosome segregation, chromosome damage, wholesale aneuploidy, or developmental arrest. To test this model, zygotes were enucleated in interphase, mitotic ES cell chromosomes transferred and the resulting cell division observed. Following nuclear transfer, these embryos entered mitosis with relatively normal kinetics. Control zygotes entered into mitosis within 31 hours post hCG (100%). At the same time point, 61 of 66 nuclear transfer zygotes (92%) were also mitotic.

To further test the ability of these nuclear transfer embryos to assemble a normal spindle, they were arrested in mitosis using nocodazole, released from the mitotic block and spindle formation observed. Within 45 min., 31/31 nuclear transfer zygotes, and 30/30 control zygotes had assembled normal bipolar spindles. These spindles were indistinguishable from those of control zygotes both by microtubule birefringence, as well as by beta-tubulin immunofluorescence staining (Fig. 2 A-F).

Similar to fertilized controls, interphase nuclear transfer zygotes proceeded from prometaphase through anaphase to cytokinesis within 90 minutes of release from the nocodazole block (Fig. 2G-I). Furthermore, there was very limited association of microtubules with the interphase pronuclei of the zygote, suggesting this component of the spindle apparatus would not be depleted by enucleation (Fig. 2D). Together, these results suggest that if components of the spindle assembly or cell division machinery are removed during interphase enucleation, there is a sufficient cytoplasmic store to allow normal cell-division.

It has been demonstrated that inhibitors of DNA replication, such as aphidicolin can arrest mouse embryos at the two-cell stage (Spindle et al., 1985). These inhibitors both arrest development and interfere with subsequent zygotic genome activation. Therefore, it seemed reasonable that enucleation of the zygote at interphase might eliminate essential components of the DNA replication machinery, preventing DNA replication in the next interphase and therefore development beyond the two-cell stage. However, we observed that both control and interphase nuclear transfer embryos (12/12) efficiently incorporated BrdU at the 2-cell stage, demonstrating that DNA synthesis could still occur (Fig. 2J, K). These results suggest that interphase nuclear transfer embryos arrest well past S-phase entry and that it is not an inability to replicate the DNA that leads to the observed developmental arrest.

Abnormal transcription following interphase nuclear transfer

Because nuclear transfer embryos enucleated in interphase proceeded through chromosome segregation, cytokinesis and DNA replication efficiently but then invariably arrested before division to the 4-cell stage, we investigated whether they underwent normal zygotic genome activation, which would be required for their subsequent development. To this end, we compared the transcriptional profile of fertilized control embryos with nuclear transfer embryos created using recipient zygotes enucleated in either interphase or in mitosis. We chose to profile embryos at the 2-cell stage because this is when ZGA occurs and because both types of nuclear transfer embryos efficiently reach the 2-cell stage, but differ in developmental potential there after. As controls, we profiled both types of enucleated recipient zygotes prior to transfer as well as un-manipulated 2-cell stage embryos and the ES donor cells.

As expected, when global gene expression patterns were compared, interphase zygotes clustered closely together with mitotic zygotes. In contrast, 2-cell stage embryos, which had undergone ZGA, had a significantly different transcriptional profile and clustered separately (Fig. 3A). Embryos subjected to nuclear transfer after enucleation in mitosis, which have high developmental potential, clustered together with control 2-cell stage embryos; this suggests that transcriptional reprogramming of the donor chromosomes had occurred. In contrast, embryos generated after enucleation in

interphase were transcriptionally distinct from other two-cell embryos, suggesting abnormalities in transcription (Fig. 3A).

The transcriptional abnormalities that followed interphase enucleation were also reflected in the number of genes that were upregulated during embryonic genome activation at the 2-cell stage. Of 13037 total transcripts that were represented on the gene expression array, we found 575 significantly ($P=0.001$) upregulated between the one and 2-cell stages, presumably reflecting zygotic genome activation (ZGA). Interestingly, 535/575 (93%, $p=0.01$) of these transcripts were also upregulated following enucleation in mitosis. In contrast, only 4/575 genes (0.7%, $p=0.01$) were properly induced in nuclear transfer embryos enucleated at interphase.

Of the 575 transcripts upregulated between the one and two cell stage, 303 were not expressed or expressed at only low levels in the donor ES cells (Fig. 3B). These were therefore transcripts from genes that would presumably require transcriptional activation following nuclear transfer. Of these 303 transcripts, 272 (90%, $p=0.01$) were properly upregulated in nuclear transfer embryos generated by mitotic enucleation. In contrast, only one gene was successfully upregulated in nuclear transfer embryos after interphase enucleation (0.3%, Nme2), which was strikingly similar to embryos that had been enucleated and then allowed to develop in the absence of chromosomes (0/303 activated).

These results indicate that the transcriptional profile of an NT embryo produced by mitotic enucleation is remarkably normal while the transcriptional profile of an NT embryo produced by interphase enucleation is abnormal and in fact, is most equivalent to that of an enucleated embryo with no chromosomes. This finding is consistent with the idea that interphase enucleation depletes the early embryo of transcriptional regulators that are required for proper zygotic genome activation from the donor chromosomes. It could be that the observed failure of germinal vesicle stage oocytes to support development following nuclear transfer was caused by the depletion of these same nuclear transcription factors (Gao et al., 2003).

In summary, enucleated interphase zygotes were unable to reprogram the ES cell genome, correlating with developmental failure. In contrast, M-phase zygotes can efficiently transition the ES cell chromosomes to a transcriptional state similar to that of a 2-cell stage embryo, correlating with a high developmental potential. Importantly, these

findings rule out the model in which insufficient time for reprogramming underlies developmental arrest following interphase nuclear transfer (Kikyo and Wolffe, 2000). Even though nuclear transfer into mitotic zygotes allows less time for reprogramming, it is still sufficient time to enable development to proceed normally.

Enucleation Depletes the Transcriptional Regulator Brg1

Our findings thus far seemed consistent with a model in which enucleation of the recipient cell in interphase not only removes the chromosomes but also depletes transcriptional regulators that are required for reprogramming. The depletion of these transcription factors might then be the cause of the observed developmental failures at the 2-cell stage. We noted that the rapid developmental arrest following interphase enucleation seemed strikingly similar to the preimplantation arrest of embryos that harbor a loss of function mutation in *Brg1* (Bultman et al., 2006). We therefore considered the possibility that interphase enucleation was depleting the early embryo of BRG1, which is a required component of the Swi/SNF chromatin remodeling complex and essential for normal zygotic genome activation.

We found that Brg1 could be readily detected by immunostaining in the maternal and paternal pronuclei of interphase zygotes, as well as in the nuclei of 2-cell stage embryos (Fig. 4A,B). However, when interphase zygotes were enucleated, the majority of Brg1 was removed from the cell (Fig. 4C). Consequently, nuclear levels of Brg1 were substantially lower in embryos that had arrested at the two cell stage following interphase enucleation; the fluorescence intensity of immuno-labeled BRG1 was reduced at least 10-fold (Fig. 4D, H). Although we did observe some residual Brg1 that originated from either RNA or cytoplasmic protein pools, the vast majority of this protein was removed by interphase enucleation. Thus the failure of interphase nuclear transfer embryos to undergo normal ZGA could be the result of depletion of the Brg1 protein and/or other transcriptional regulators.

A hallmark of mitotic entry is breakdown of the nuclear envelope and dispersion of many nuclear factors through out the cytoplasm, which allows the two resulting daughter cells to inherit equal portions of nuclear components. When the localization of Brg1 was assessed in mitotic zygotes, we found that it too was scattered throughout the

cytoplasm and excluded from the chromatin (Fig. 4E,F). The cell-cycle dependence of Brg1 localization we observed was consistent with that previously reported in somatic cells and in mouse oocytes, where Brg1 localizes to the interphase nucleus, but is dispersed in the cytoplasm during mitosis (Muchardt et al., 1996; Sun et al., 2007). As a result, when recipient cell chromosome extraction was performed after mitotic entry, Brg1 was not depleted and the resulting 2-cell embryos (Fig. 4G) had Brg1 levels comparable to the two-cell control embryos (Fig. 4B,H) and developed normally. Thus the removal of Brg1, and likely many other transcription factors, with the interphase nucleus correlated with developmental failure, while the retention of these factors correlated with normal development and successful transcriptional reprogramming.

Factors required for reprogramming associate closely with chromatin in interphase but not in mitosis

We next considered whether or not performing interphase enucleation via a method that would allow the zygote to maintain a subset of its nuclear factors would stimulate its capacity to develop after nuclear transfer. Recently, a novel method for interphase enucleation has been developed. Instead of aspirating the entire nucleus from the zygote, the nucleus is mechanically disrupted, and the nuclear envelope with attached chromatin is more specifically removed (see Fig. S3A in the supplementary material) (Greda et al., 2006). This disruption of the nuclear envelope might be expected to release some nuclear components into the cytoplasm, allowing them to be left behind after removal of the chromatin.

We removed the chromosomes from interphase zygotes by either conventional enucleation or by mechanically disrupting the nucleus prior to removing the chromatin. We then transferred nuclei or mitotic chromosomes from various donor cell types into these recipients and compared the extent and efficiency of development (Fig. 5A-F). As had been previously reported (McGrath and Solter, 1984), when 8-cell stage donor nuclei were injected into normally enucleated zygotes, they failed to develop. In contrast, when these blastomere donor nuclei were introduced into zygotes whose nuclei had been mechanically disrupted prior to enucleation, these embryos developed to the blastocyst stage (Greda et al., 2006)(Fig. 5E).

However, when more differentiated ES cells were used as nuclear donors, development invariably arrested at the 2-cell stage, regardless of whether or not enucleation was carried out with disruption of the nuclear envelope (Fig. 5B). In these experiments, as before, development only proceeded when ES cell donor chromosomes were injected into control mitotic zygotes (Fig. 5B,G).

The development of embryos after transfer of 8-cell stage nuclei into specifically enucleated interphase nuclei suggests that disruption of the nuclear envelope prior to enucleation releases some factors required for development. However, this release must not be complete as it is not sufficient to support transcriptional reprogramming of ES cell chromosomes.

Consistent with this interpretation, when one of the pro-nuclear envelopes was disrupted by micromanipulation, a modest amount of Brg1 was released into the cytoplasm. However, the broken pro-nucleus still retained significant quantities of this protein after its removal (n=5/5) (Fig. 6). Our findings demonstrate that activities required for transcriptional reprogramming are in part closely associated with chromatin in interphase and that they are removed with the interphase chromosomes regardless of the enucleation method used. In contrast, these nuclear factors are not closely associated with mitotic chromosomes, allowing the maximal concentration of these factors to be retained when the recipient cell chromosomes are removed during mitosis (Fig. 5E-G).

Removal of interphase nuclei depletes factors required for ZGA

The depletion of Brg1 caused by interphase enucleation and the immediate developmental arrest that resulted raised the question of whether the transcriptional regulators depleted by interphase enucleation were specifically required for reprogramming or whether they are also more generally required for processes that facilitate preimplantation development, for instance ZGA. To distinguish between these two models, we transferred chromosomes from one mitotic donor zygote into a recipient zygote that had been enucleated in interphase. The resulting nuclear transfer embryos should not require reprogramming activities as the transferred chromosomes are derived from exactly the same cell type. However, these embryos would still be expected to require all activities necessary for normal preimplantation development.

When mitotic donor chromosomes from one zygote were transferred into another zygote that had been enucleated in interphase, the resulting embryos invariably arrested at the 2-cell stage (Fig. 5D,G). These embryos formed small nuclei without the prominent nucleoli usually present at this stage (see Fig. S3b in the supplementary material).

To control for these manipulations, interphase nuclei from one zygote were transferred into recipient zygotes enucleated in that same phase of the cell cycle. As expected, and previously observed, (McGrath and Solter, 1983), these control embryos developed very efficiently (Fig. 5F,G). Similarly, when mitotic chromosomes from a zygote were transferred into an interphase zygote that had been enucleated following nuclear disruption, the resulting embryos could develop to term, albeit at a low efficiency (Fig. 5D,G, see Table S2 in the Supplementary Material). Removal of chromatin in interphase after nuclear envelope disruption therefore depletes, but does not completely eliminate, factors required for development. This depletion does however severely compromise the ability of the zygote to reprogram more differentiated cells.

Factors from embryonic nuclei can partially replace those depleted by interphase enucleation

If enucleation removes factors required for normal development and reprogramming, then re-introduction of these factors might be expected to restore developmental potential. We reasoned that nuclei, but not mitotic chromosomes, of cells developmentally related to the zygote might provide a source of these factors. Consistent with this idea, we found that the nuclei of cleavage stage embryos contained high levels of Brg1, and that Brg1 was excluded from the condensed chromatin when these cells entered mitosis (Fig. 7). We isolated either nuclei or mitotic chromosomes from 8-cell stage blastomeres and then injected them into zygotes enucleated in interphase by the conventional method. Injection of the mitotic 8-cell stage chromosomes resulted in arrest at the 2-cell stage (Fig. 5C) while transfer of the 8-cell interphase nucleus and its accompanying factors allowed further development to a compacted 4-cell stage (Fig. 5E).

To determine whether the factors in the 8-cell stage nucleus could collaborate with a reduced dose of zygotic nuclear factors to direct development, either nuclei or mitotic chromosomes from 8 cell stage blastomeres were transferred into zygotes that had

their nuclei disrupted prior to enucleation. As previously described, when 8-cell stage nuclei were transferred into these cytoplasts, embryonic development to the blastocyst stage was observed (Fig. 5E). However, when mitotic chromosomes from the same blastomeres were transferred, development failed (Fig. 5C). Thus factors contained in the 8-cell stage nucleus can partially complement the effect of losing the factors removed from the zygote along with the interphase nucleus. However, this complementation is not complete because if the zygote pronuclei are fully removed and then replaced with a blastomere nucleus, development cannot proceed (see Table S2 in the supplementary material). Thus, the quantities of Brg1 in the 8-cell stage nucleus are either insufficient or it is not the only limiting factor removed along with the zygotic pronuclei.

Discussion

Our findings demonstrate that reprogramming activities are not degraded after fertilization, but persist throughout the first cell cycle in the zygote and even in 2-cell stage blastomeres (Egli et al., 2009). We also show that the early arrest of nuclear transfer embryos after interphase microinjection are not always the direct result of incompatibilities between the cell cycle of the zygote and the donor cell, nor a result of a defect in chromosome segregation during mitosis. Instead, our experiments suggest these phenotypes are secondary consequences of the depletion of nuclear factors caused by interphase enucleation.

Our analysis here suggests that the essential nuclear factors removed by enucleation are transcriptional regulators required for zygotic genome activation and reprogramming. It is likely that components of the DNA replication and spindle assembly machinery that associate with chromatin in interphase but dissociate from it in mitosis (Cuvier et al., 2008) are also removed with the interphase nucleus. However, zygotes enucleated in interphase maintain the ability to form a functional bi-polar spindle and to replicate their DNA, suggesting that depletion of these factors is not the causes for developmental arrest. Instead, failure to develop further is likely to be a direct consequence of the highly abnormal gene expression patterns we observed.

In these studies we were surprised by the flexibility of cell cycle parameters for successful nuclear transfer. Zygotes were suitable genome recipients in mitosis as well as in interphase and cell cycle synchrony between donor and recipient was not strictly required at the time of transfer. In some experiments, the mitotic donor genome was initially asynchronous with the interphase zygote and became synchronous only upon entry of the zygote into the first mitosis. With the exception of perhaps donor cells in S-phase, there appear to be few constraints on the recipient or donor-cell cycle requirements for successful reprogramming (Campbell et al., 1996a).

Our finding that reprogramming and development after nuclear transfer succeeds as long as nuclear components are retained is consistent with results from other reprogramming systems. When somatic cells are fused with either intact ES cells or isolated ES cell nuclei, transcriptional reprogramming of the somatic chromosomes occurs and stable hybrid cell lines can be established (Cowan et al., 2005; Do and Scholer, 2004; Tada et al., 2001). However, when ES cells were enucleated in interphase, they could no longer reprogram the somatic genome (Do and Scholer, 2004).

The four reprogramming transcription factors required for the generation of iPS cells (Takahashi and Yamanaka, 2006), Oct4, Klf4, cMyc and Sox2, each localize to the nuclei of interphase ES cells. Thus, just as we have shown here, depletion of these nuclear factors after ES cell enucleation is a plausible explanation for the failure of reprogramming approaches based on cell fusion. This suggests that removal of the ES cell chromosomes in mitosis might result in an ES cell derived cytoplasm that retained reprogramming potential.

Also consistent with our model, injection of nuclei directly into the germinal vesicle (nucleus) of the frog oocyte results in reprogramming of transcription (Byrne et al., 2003), while removal of the germinal vesicle from mouse oocytes depletes the oocyte of reprogramming activities (Do and Scholer, 2004; Gao et al., 2002; Pralong et al., 2005).

All of these findings support the view that nuclear components, particularly those required for transcriptional regulation, are critical for reprogramming and must be retained in the early embryonic recipient cell if development is to proceed. Specifically illustrating our point, we found that the essential transcriptional regulator Brg1 is

sequestered in the interphase nucleus of a zygote but becomes dispersed throughout the cytoplasm during mitosis

We found that this cell-cycle dependent distribution of Brg1 contributed to its depletion when either interphase nuclei or interphase chromatin were removed from the recipient cell. The depletion of Brg1 was associated with failures in reprogramming and a developmental arrest similar to that observed in mutant embryos engineered to lack this protein (Bultman et al., 2006). In contrast, Brg1 protein was preserved in the cell when the chromatin was removed in mitosis, facilitating both development and reprogramming of gene expression.

However, our experiments suggest that Brg1 is not likely to be the only factor depleted by interphase enucleation. Many other transcriptional regulators undergo a similar redistribution in mitosis and they would also be depleted by interphase enucleation. In interphase, transcription factors localize to the nucleus, where they are in intimate association with the chromatin, regulating gene expression. When cells enter into mitosis, gene expression becomes repressed and these proteins are dispersed throughout the cytoplasm, allowing them to be equally inherited by each daughter cell (Egli et al., 2008; Gao et al., 2007; Gottesfeld and Forbes, 1997; Martinez-Balbas et al., 1995; Sun et al., 2007). We propose that it is the sum of these transcriptional regulators in a particular cell that define its cell-type specific gene expression pattern as well as the transcriptional program that is engaged after its use as a recipient cell in nuclear transfer.

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Figure Legends

Fig. 1. Development after mitotic genome transfer into interphase zygotes.

(A) Diagram of the transfer of a mitotic ES cell genome (red) into an H2B-GFP positive (green) zygote in interphase. (B) Zygote immediately after transfer. (C,D) zygote in mitosis in the presence of nocodazole. Note the difference in intensity of green and red histones on the two zygotic (haploid maternal and haploid paternal (arrows), and the diploid ES cell genome (arrowhead). (E) Removal of zygotic nuclei before entry into mitosis, while still in interphase. (F) Removal of nocodazole from mitotic zygotes results in the assembly of both ES cell genome and zygotic genomes in a single spindle (schematic). (G-I) Development of tetraploid embryos with both ES cell genome and zygotic genome expressing both H2B-cherry and H2B-GFP. (J) removal of zygotic genomes. (K-N) Development of embryos after the removal of the zygotic genome. Images in (G-I) and (K-N) were taken with identical exposure time. (O) Development to the morula and blastocyst stage after genome transfer into zygotes in interphase. Numbers indicated the number of morulas and blastocysts of successfully transferred (cleaved) embryos. PB= polar body. (For detailed results see Table S1 in the Supplementary Material.)

Fig. 2. Removal of interphase nuclei permits spindle assembly and replication in nuclear transfer embryos

(A-C) Control zygotes in mitosis. (A) shortly after release from 0.4µg/ml nocodazole. (B), and immunocytochemistry (C). (D) Zygote in mitosis prior to removal of interphase

nuclei, in the presence of 0.4µg/ml and 10µg/ml cytochalasinB used for enucleation. **(E-I)** Mitotic progression of zygotes after removal of interphase nuclei and subsequent transfer of a mitotic ES cell genome. BrdU incorporation in control **(J)** and interphase nuclear transfer embryos **(K)**.

Fig. 3. Nuclear factors are required for reprogramming

(A) Gene expression cluster analysis of nuclear transfer and control embryos: interphase zygotes were harvested 25-28h post hCG injection, mitotic zygotes were obtained by nocodazole mediated arrest until 33h post hCG. Nuclear transfer embryos (below the line) were allowed to the 2-cell stage and harvested 22-24h post transfer. Each datapoint consists of two samples. Two independent experiments (2 and 2 samples each) of ES cells transferred into M-phase zygotes are shown. **(B)** heat map of genes upregulated at the 2-cell stage and not expressed in the donor cell (left), or downregulated at the 2-cell stage and expressed in ES donor cells (right). Shown is the differential gene expression score relative to M-phase zygotes.

Fig. 4. BRG1 is associated with chromatin in interphase zygotes and excluded in mitosis

(A) A zygote in interphase. **(B)** 2-cell stage un-manipulated control embryo. **(C)** Zygote nucleus removed from an interphase zygote. **(D)** 2-cell stage embryo after enucleation in interphase and transfer of a mitotic ES cell genome. One of the two cells are shown at high magnification (note size bars). **(E)** M-phase zygote arrested with nocodazole. **(F)** Zygote without genome and removed nuclear material (arrow) of an M-phase zygote arrested in nocodazole. **(G)** 2-cell stage embryo after genome removal in mitosis and transfer of a mitotic ES cell genome. **(H)** Relative quantification of BRG1 nuclear staining. Interphase and mitosis indicate the time point of enucleation. Error bars represent the standard deviation of at least 3 different cells.

Fig. 5. Developmental outcome is determined by the removal and transfer of developmental factors

(A) Recipient zygotes are prepared either by complete enucleation in interphase, by breaking the nuclear envelope prior to removal of the interphase chromatin or by extraction of mitotic chromosomes. Each of these genome-less zygotes is transferred with an entire mitotic ES cell (B), or an isolated mitotic 8-cell stage genome (C), or a mitotic zygotic genome (D), or an 8-cell stage interphase nucleus (E) or an interphase nucleus of a zygote (F). The green color indicates the distribution of putative nuclear factors involved in preimplantation development. (G) Percentage of embryos developing to the morula and blastocyst stages. Numbers indicated the number of morulas and blastocysts of successfully transferred (cleaved) embryos. (For detailed results see Table S2 in the Supplementary Material.) Size bar represents 20µm. The broken lines in E, top lane, represent the boundaries between different blastomeres.

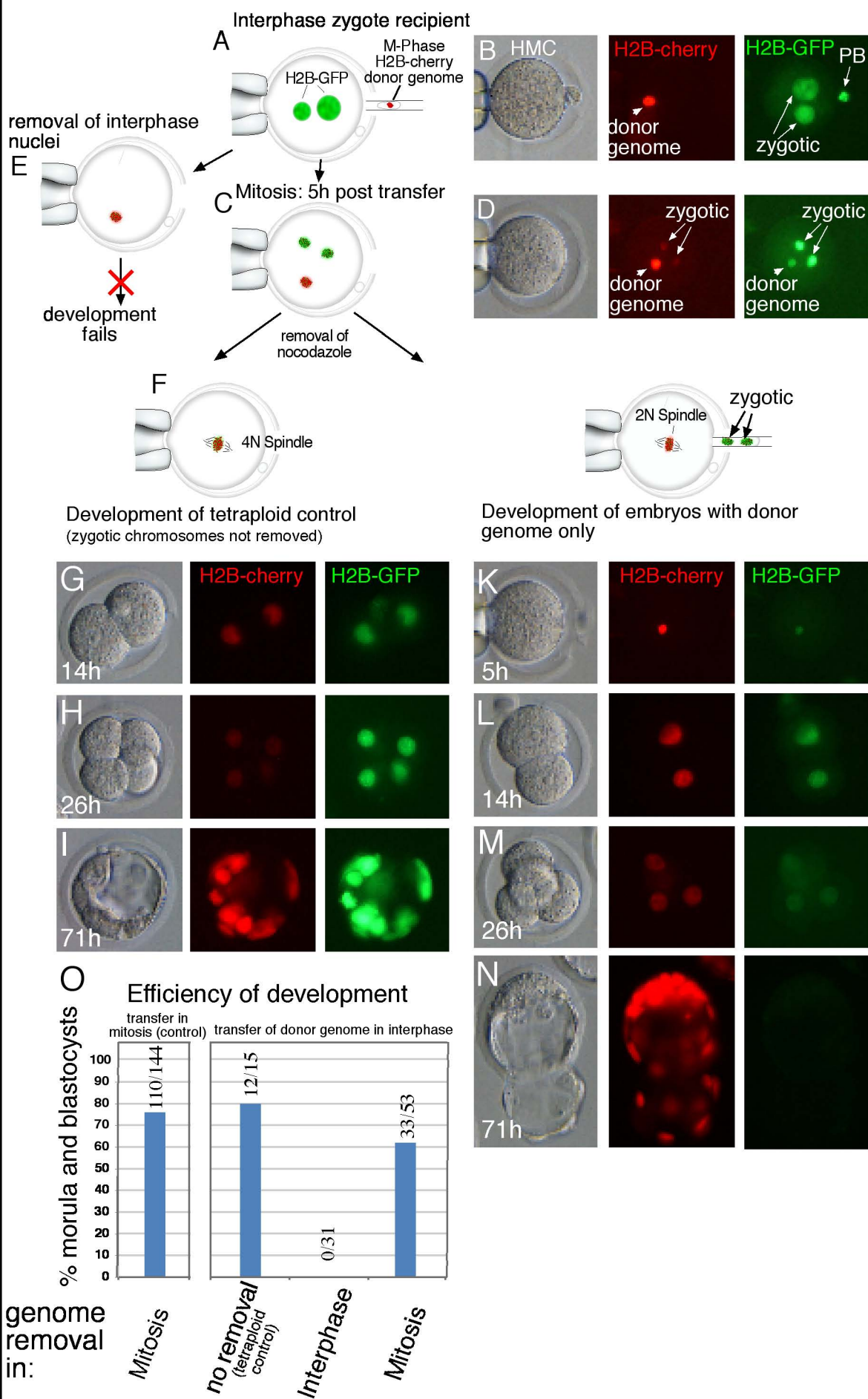
Fig. 6. BRG1 is associated with chromatin

(A) A zygote in interphase with a nucleus broken by micromanipulation (star*) and an intact pronucleus (arrow). Note the presence of BRG1 on the chromatin even in the broken nucleus, albeit at a lower intensity than of the intact nucleus. (B) relative quantification of BRG1 nuclear staining. Error bars represent the standard deviation of at least 3 different cells.

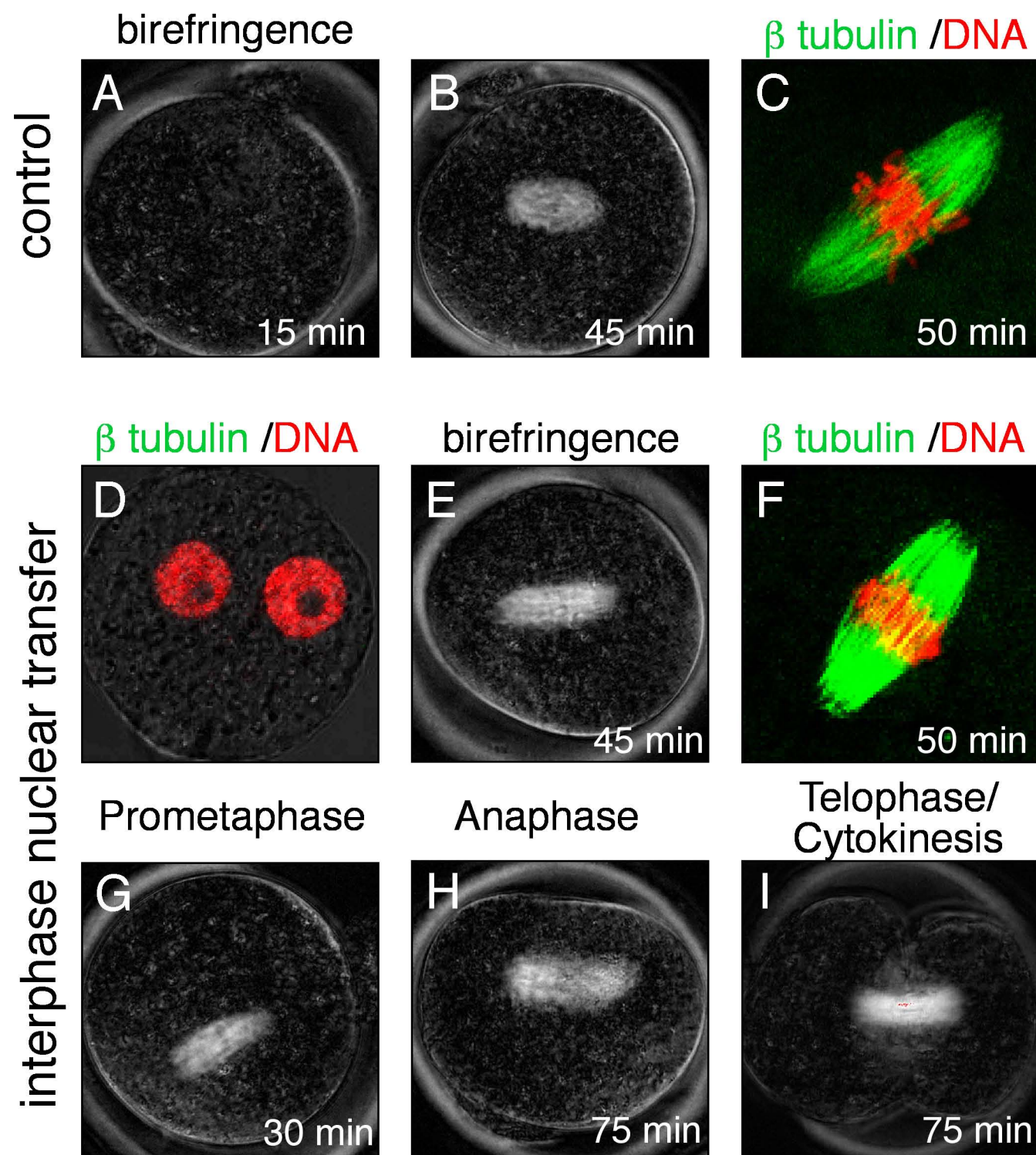
Fig. 7. BRG1 is found in interphase nuclei of blastomeres, but not on their mitotic chromosomes

Nuclei of blastomeres of a cleavage stage embryo in interphase and in mitosis (arrow). Again note the absence of BRG1 from mitotic chromatin.

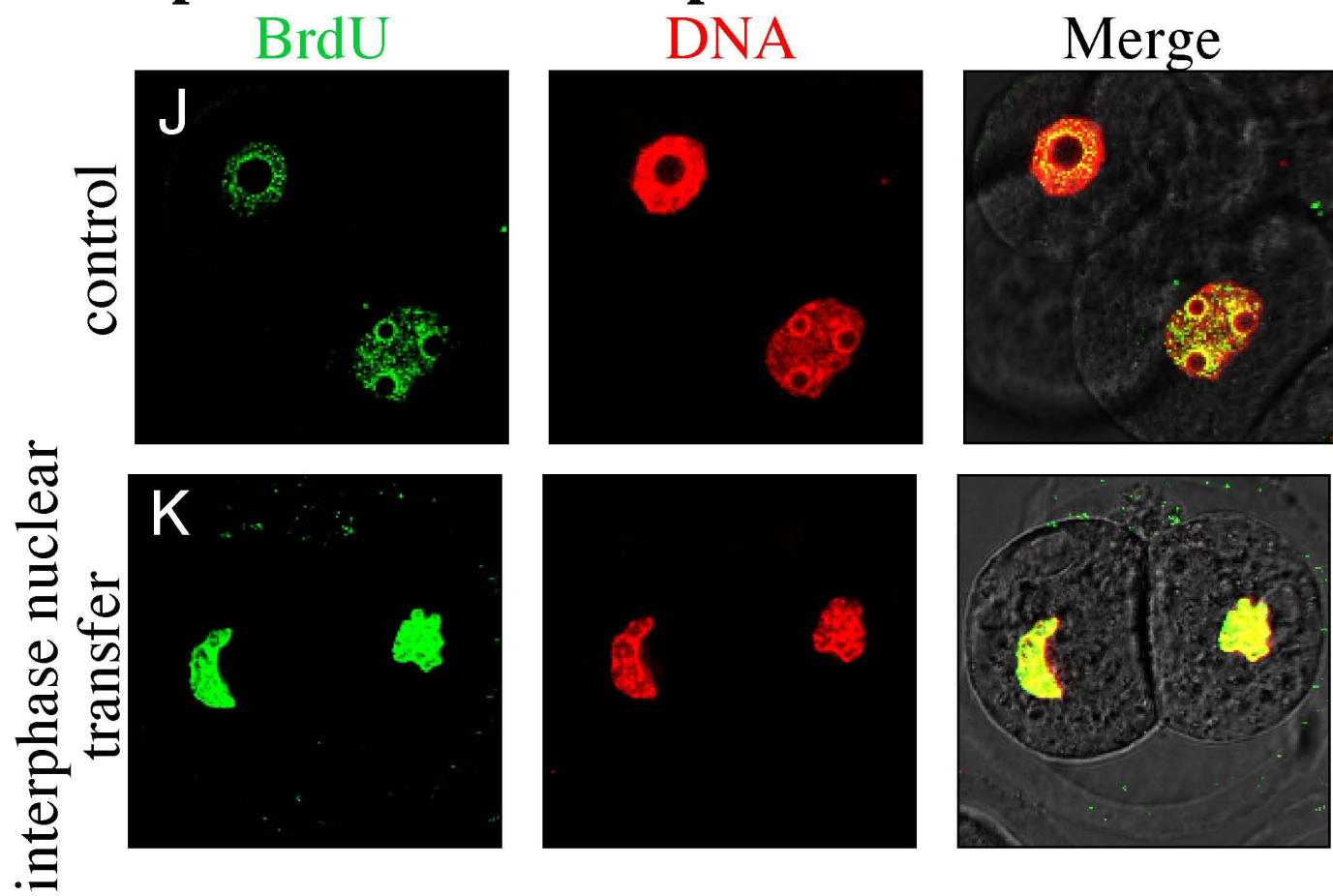
Development after genome transfer into interphase recipient



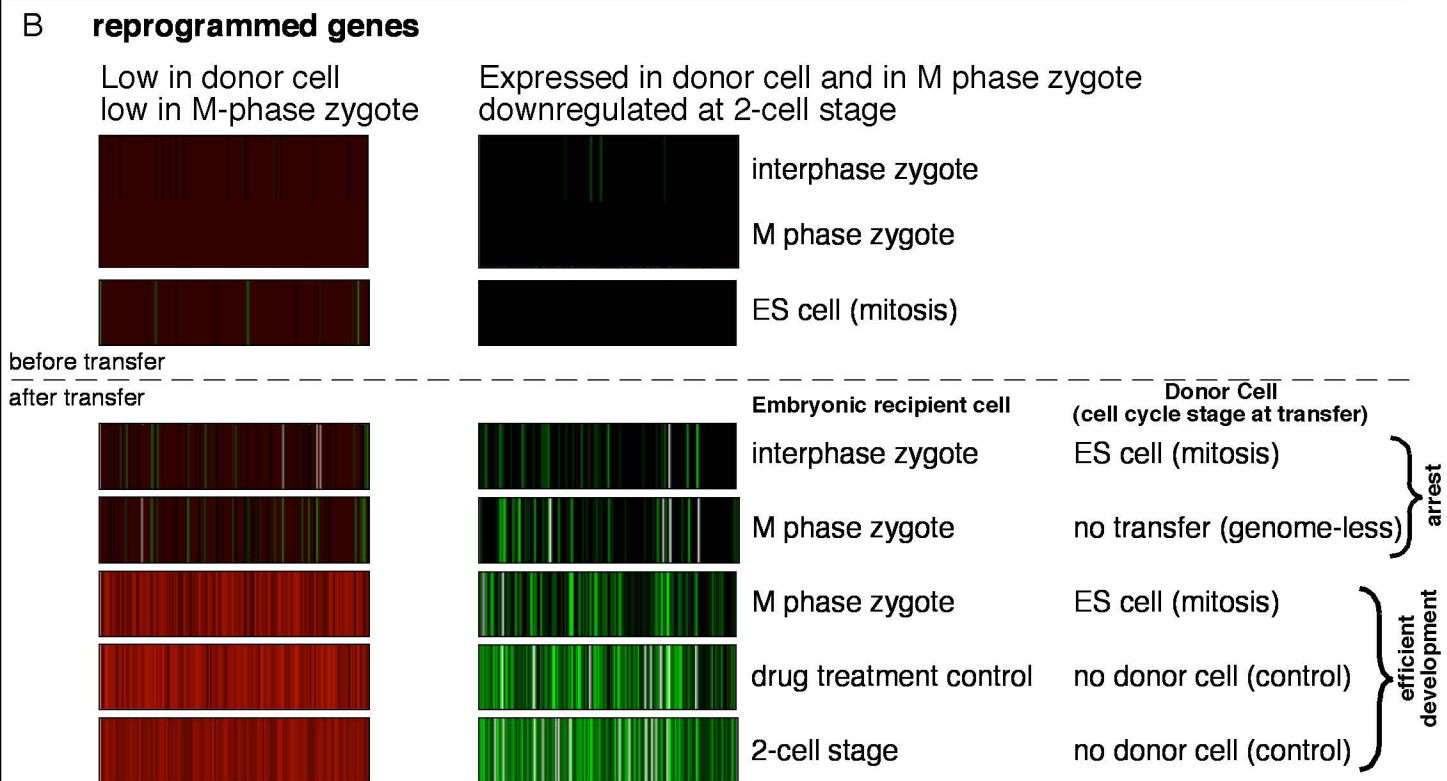
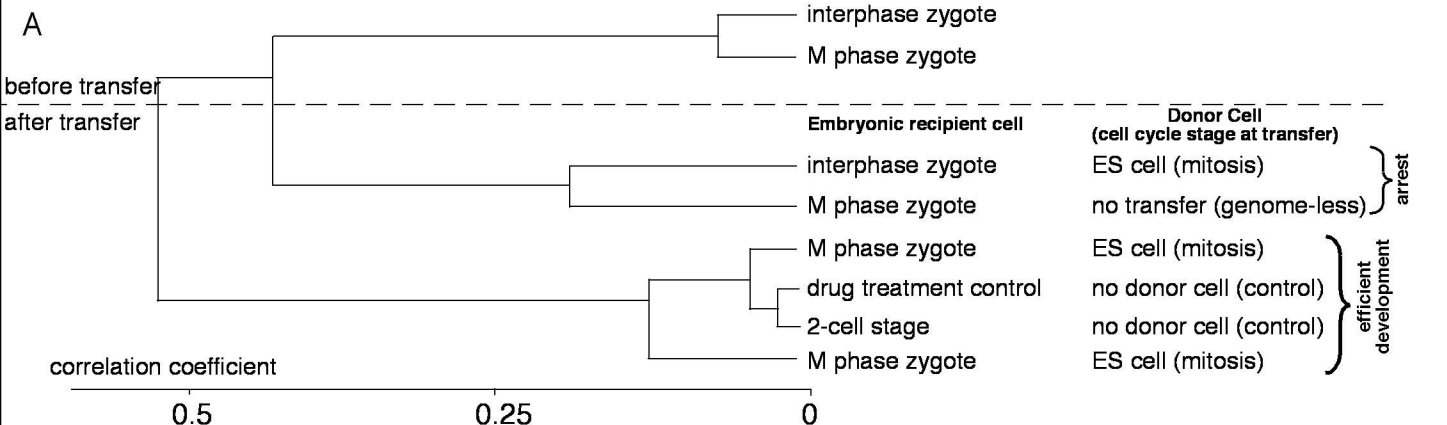
Spindle assembly after interphase nuclear transfer



Replication after interphase nuclear transfer



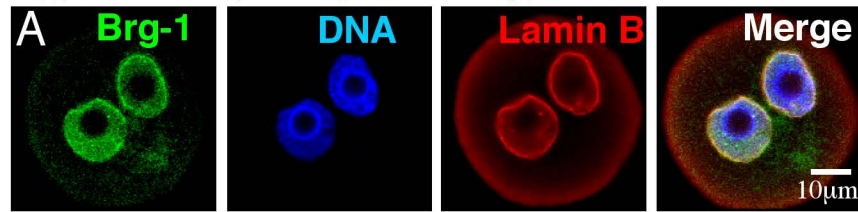
Mitotic, but not interphase zygotes reprogram gene expression of an ES donor cell



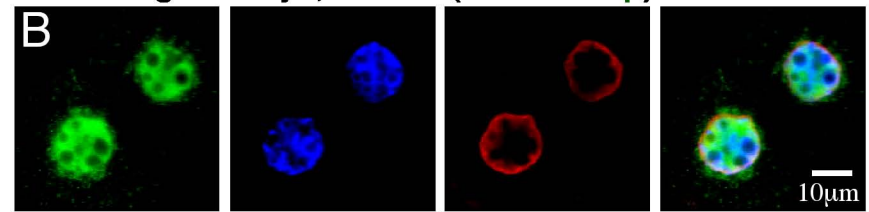
Localization of Brg-1 in nuclear transfer embryos

Egli, Figure 4

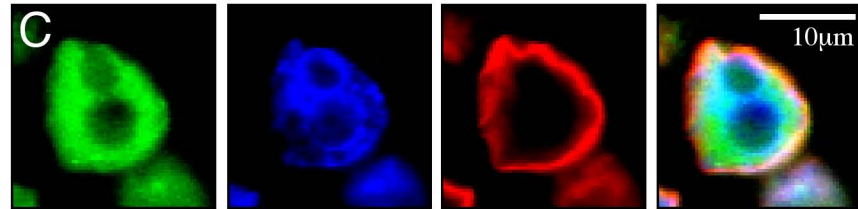
Zygote in interphase (will develop)



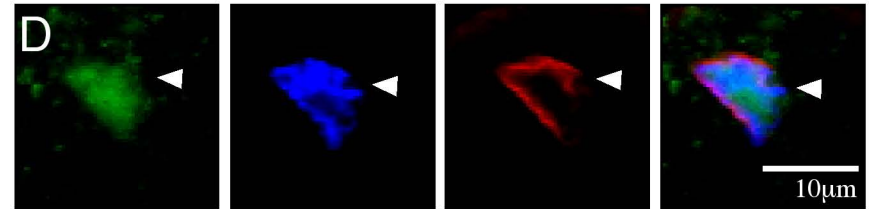
2-cell stage embryo, control (will develop)



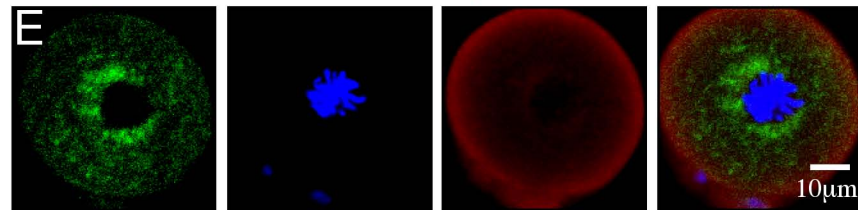
Nucleus extracted from interphase zygote



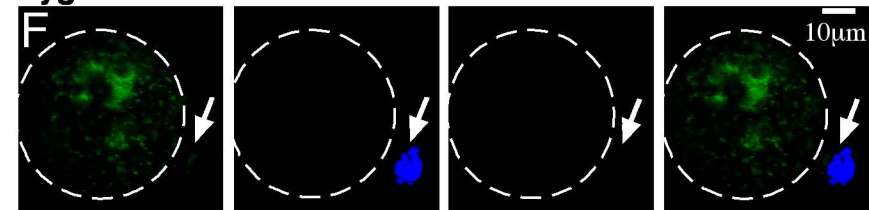
2-cell stage nuclear transfer embryo, zygotic genome removed in interphase (will not develop)



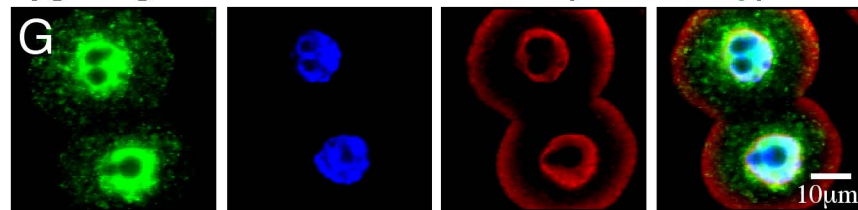
Zygote in M-phase (will develop)



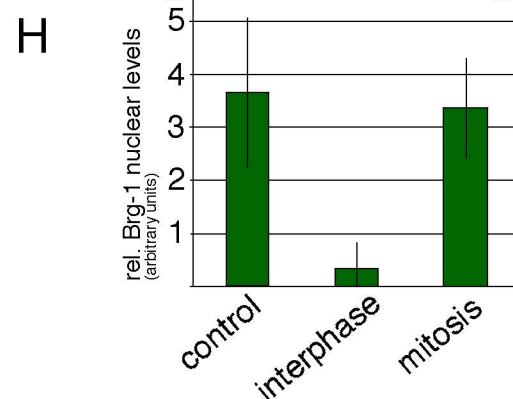
Zygote after mitotic chromosome extraction

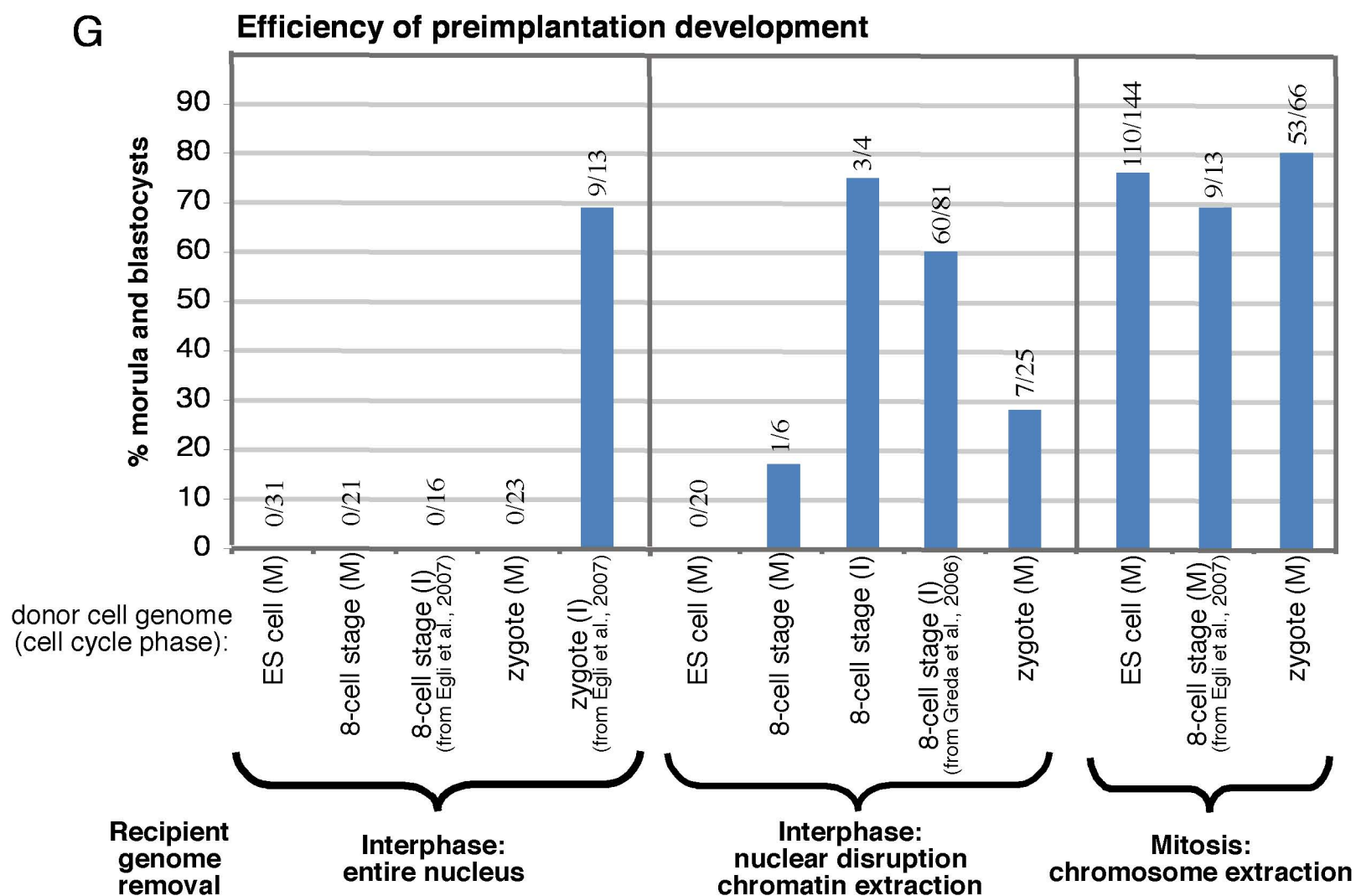
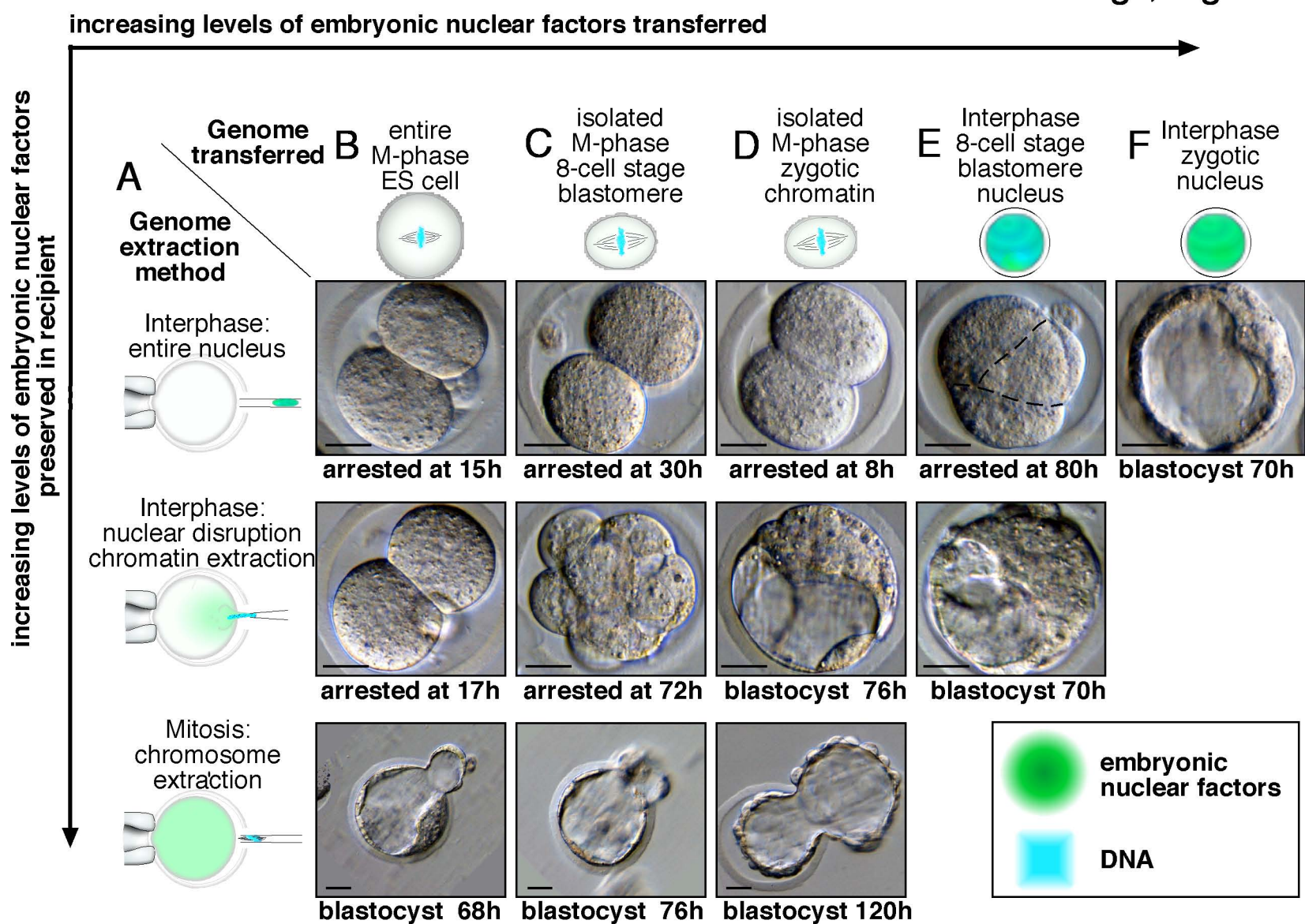


2-cell stage nuclear transfer embryo, zygotic genome removed in mitosis (will develop)

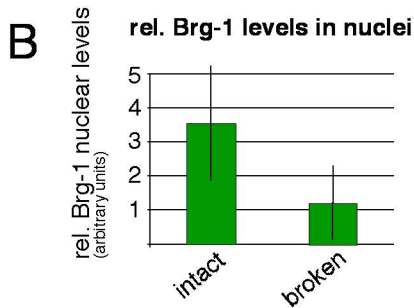
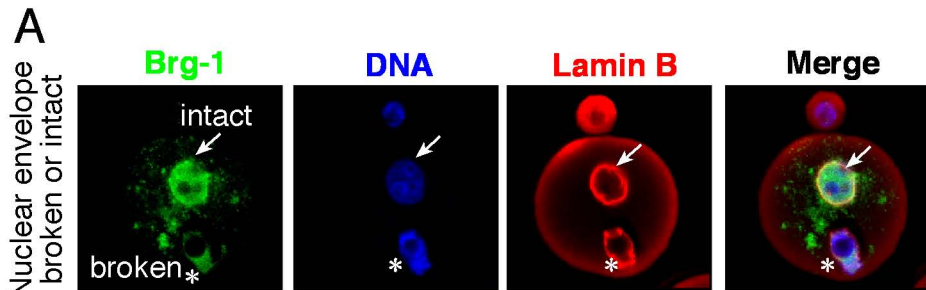


rel. Brg-1 levels in 2-cell stage embryos



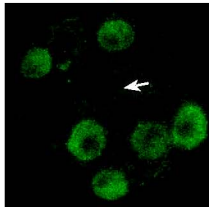


Brg-1 stably associates with chromatin in interphase

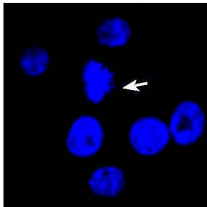


Brg-1 in cleavage stage embryos

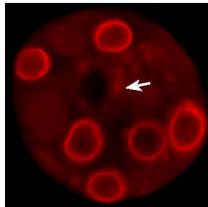
Brg-1



DNA



Lamin B



Merge

